Comparison of the cyclooxygenase-1 inhibitory properties of nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors, using sensitive microsomal and platelet assays

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Abstract: Two forms of cyclooxygenase (COX) activity are involved in the synthesis of prostaglandins, prostacyclins, and thromboxanes in mammalian cells. There is now convincing evidence, obtained with a number of structurally distinct inhibitors, that selective COX-2 inhibitors possess anti-inflammatory effects with an improved gastrointestinal tolerability compared with conventional nonsteroidal anti-inflammatory drugs (NSAIDs) affecting both COX-1 and COX-2. As more selective COX-2 inhibitors are being developed, assays with a high degree of sensitivity to inhibition are needed to compare the relative effects of compounds on COX-1 activity. In the present report, we describe a sensitive assay for the inhibition of human COX-1 based on the production of prostaglandin E_2 by microsomes from U937 cells incubated with a subsaturating concentration of arachidonic acid. More than 45 NSAIDs and selective COX-2 inhibitors were tested in this assay. IC_{50} values ranged from 1 nM for flunixin and flurbiprofen to about 200–500 μM for salicylate and acetaminophen. Potent and nonselective NSAIDs such as sulindac sulfide, diclofenac, and indomethacin showed IC_{50} values of <20 nM. Among the compounds that have been reported to show selectivity for COX-2, the rank order of potency against COX-1 was DuP 697 > SC-58451 > celecoxib > nimesulide ~ meloxicam ~ piroxicam ~ NS-398 ~ RS-57067 > SC-57666 > SC-58125 > flosulide > etodolac > L-745,337 > DFU ~ T-614, with IC_{50} values ranging from 7 nM to 17 μM. A good correlation was obtained between the IC_{50} values for the inhibition of microsomal COX-1 and both the inhibition of TXB_2 production by Ca^{2+} ionophore challenged platelets and the inhibition of prostaglandin E_2 production by CHO cells stably expressing human COX-1. However, the microsomal assay was more sensitive to inhibition than cell-based assays and allowed the detection of inhibitory effects on COX-1 for all NSAIDs and selective COX-2 inhibitors examined with discrimination of their potency under conditions of limited availability of arachidonic acid.

Key words: cyclooxygenase, prostaglandin synthase, nonsteroidal anti-inflammatory drugs, COX-2 inhibitors, platelets, gastrointestinal toxicity.

Résumé : Deux formes d'activité de la cyclo-oxygénase (COX) participent à la synthèse des prostaglandines, des prostacyclines et des thromboxanes dans les cellules des mammifères. Des faits probants, obtenus au moyen de divers inhibiteurs de structures distinctes, indiquent que les inhibiteurs sélectifs de la COX-2 possèdent des effets anti-inflammatoires offrant une plus grande tolérance gastro-intestinale que les anti-inflammatoires non stéroïdiens conventionnels (AINS) affectant tant COX-1 que COX-2. Avec la mise au point d'inhibiteurs plus sélectifs de la COX-2, on doit envisager l'emploi d'essais plus sensibles à l'inhibition pour comparer les effets relatifs des composés sur l'activité de la COX-1. Dans la présente étude, nous décrivons un essai sensible pour évaluer l'inhibition de la COX-1 humaine; cet essai est basé sur la production de prostaglandine E_2 par les microsomes de cellules U937 incubés avec une concentration sous-saturante d'acide arachidonique. Plus de 45 AINS et inhibiteurs sélectifs de la COX-2 ont été testés dans cet essai. Les valeurs de IC_{50} étaient comprises entre 1 nM pour le flunixin et le flurbiprofène et 200–500 μM pour le salicylate et l'acétaminophène. Des AINS non sélectifs et plus puissants, comme le sulindac, le diclofénac et l'indométacine, ont montré des valeurs de IC_{50} < 20 nM. Parmi les composés ayant montré une sélectivité pour COX-2, l'ordre de puissance contre COX-1 a été le suivant : DuP 697 > SC-58451 > célécoxib > nimésulide ~ méloxicam ~ piroxicam ~ NS-398 ~ RS-57067 > SC-57666 > SC-58125 > flosulide > étodolac > L-745,337 > DFU ~T-614, avec des valeurs de IC_{50} comprises entre 7 nM et 17 μM. Une bonne corrélation a été obtenue entre les valeurs de IC_{50} pour l'inhibition de la COX-1 microsomale et l'inhibition de la production de thromboxane B_2 par les plaquettes stimulées à l'ionophore du Ca^{2+}, et celles pour l'inhibition de la production de prostaglandine E_2 par les cellules CHO exprimant de manière stable la COX-1 humaine. Toutefois, l'essai

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microsomal est plus sensible à l'inhibition que les essais cellulaires, et il permet de détecter les effets inhibiteurs sur la COX-1 de tous les AINS et inhibiteurs sélectifs de la COX-2 examinés, et d'évaluer leur puissance dans des conditions de disponibilité limitée d'acide arachidonique.

Mots clés : cyclo-oxygénase, prostaglandine synthase, anti-inflammatoires non stéroïdiens, inhibiteurs de COX-2, plaquettes, toxicité gastro-intestinale.
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Introduction

Cyclooxygenase (COX) catalyses the oxygenation of arachidonic acid to prostaglandin (PG) H_2 as the first step in the synthesis of prostaglandins, prostacyclins, and thromboxanes in mammalian cells. COX-1 is the major enzyme form found in healthy tissues and plays a role in thrombogenesis and in the homeostasis of the gastrointestinal tract and kidneys. COX-2 is inducible by endotoxin, cytokines, and mitogens and has been associated with the elevated production of prostaglandins observed during inflammation, pain, and pyretic responses (Bakhle and Botting 1996; Herschman 1996; Vane and Botting 1995).

Most of the classical nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, ibuprofen, or diclofenac inhibit both COX-1 and COX-2 with little or no selectivity for a particular cyclooxygenase isoform (Battistini et al. 1994; O'Neill et al. 1994). It is now well recognized that the administration of NSAIDs to animals results in gastrointestinal lesions and that the occurrence of gastrointestinal ulceration and bleeding is a major side effect associated with the chronic use of NSAIDs (Allison et al. 1992; Langman et al. 1994; Traversa et al. 1995). These effects have been attributed to the inhibition of the synthesis of prostaglandins derived from COX-1, resulting in alterations of the regulation of mucosal blood flow, mucous and bicarbonate secretion, and tumor necrosis factor-alpha (TNFα) production. Other factors, such as topical irritancy and enterohepatic recirculation of the drugs, have also been considered in the process of NSAID-induced gastropathy (Appleyard et al. 1996; Hudson et al. 1992; Rainsford 1992; Whittle 1992). Additional evidence for the implication of COX-1 inhibition in NSAID-induced gastric damage has been provided by recent studies with selective COX-2 inhibitors demonstrating that these compounds are effective anti-inflammatory agents with a marked decrease of ulcerogenicity in healthy animals compared with known NSAIDs (Chan et al. 1995; Futaki et al. 1994; Gans et al. 1990; Masferrer et al. 1994; Riendeau et al. 1997). Current clinical studies with the selective COX-2 inhibitors celecoxib (Hubbard et al. 1996) and MK-966 (Ehrich et al. 1996) should allow the evaluation of the therapeutic advantages of selective COX-2 inhibition.

Previous studies with the selective COX-2 inhibitors NS-398, DuP 697, and DFU have shown that these compounds are time-dependent inhibitors of COX-2 and rapidly reversible competitive inhibitors of COX-1 (Copeland et al. 1994; Ouellet and Percival 1995; Riendeau et al. 1997). Assays with recombinant enzymes are typically performed using arachidonic acid concentrations ranging from 1 to 10 μM to evaluate inhibitor selectivity (Gierse et al. 1995; Laneuville et al. 1995; Leblanc et al. 1995). Several of the selective COX-2 inhibitors show no inhibitory effects on COX-1 activity under these conditions, with the detection of inhibition at high doses being sometimes limited by compound insolubility in aqueous media. We have previously reported that inhibition of COX-1 activity by selective COX-2 inhibitors can be detected using microsomes from U937 cells incubated with a low concentration of arachidonic acid (Riendeau et al. 1997). In this study, the inhibitory effects of a large number of NSAIDs and COX-2 inhibitors on COX-1 activity were evaluated under conditions of limited arachidonic acid availability and were compared with those observed with platelet and whole-cell assays.

Materials and methods

NSAIDs and COX-2 inhibitors

Acetaminophen, salicylic acid, isoxicam, niflumic acid, carprofen, phenylbutazone, meclofenamic acid, mefenamic acid, flufenamic acid, nabumetone, diclofenac, fenoprofen, ketoprofen, nimesulide, piroxicam, naproxen, zomepirac, etodolac, and ibuprofen were obtained from Sigma Chemicals (St. Louis, Mo.). Indomethacin, acetylsalicylic acid, and flurbiprofen were purchased from Cayman Chemicals, Ann Arbor, Mich. Flunixin (banamine) was obtained from Schering-Plough, Pointe-Claire, Que., and ketorolac from Roche Bioscience, Mississauga, Ont. Tenidap (Moore et al. 1996) was provided by the Central Research Division, Pfizer, Groton, Conn. Sulindac sulfide, azapropazone, fenclofenamic acid, benoxaprofen, tepoxalin, NS-398 (Futaki et al. 1994), DuP 697 (Gans et al. 1990), flosulide (CGP 28238) (Wiesenberg-Bottcher et al. 1989), meloxicam (Engelhardt et al. 1996), 6-methoxy-2-naphthaleneacetic acid (6-MNA) (Blower 1992), SC-58451 (Reitz et al. 1995), SC-58125 (Seibert et al. 1994), SC-57666 (Reitz et al. 1994), L-745,337 (Chan et al. 1995), 6-[5-(4-chlorobenzoyl)-1,4-dimethyl-1*H*-pyrrol-2-ylmethyl]-2*H*-pyridazin-3-one (RS-57067) (Barnett et al. 1996), T-614 (Tanaka et al. 1995), celecoxib (Hubbard et al. 1996), L-745,296 (compound 23, Leblanc et al. 1995), and 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5*H*)-furanone (DFU) (Riendeau et al. 1997) were synthesized in the Department of Medicinal Chemistry, Merck Frosst Centre for Therapeutic Research, Canada, or were obtained from Merck Research Laboratories, Rahway, N.J.

Inhibition of microsomal COX-1 at low arachidonic acid concentration

U937 cells were obtained from the American Type Culture Collection and cultured in spinner flasks in RPMI (Sigma Chemical) supplemented with 2 g/mL $NaHCO_3$, 50 IU/mL penicillin, 50 μg/mL streptomycin, and 10% heat inactivated fetal bovine serum. These cells express COX-1 and no detectable amounts of COX-2 by reverse transcriptase – polymerase chain reaction (RT-PCR) and immunoblot analyses (Wong et al. 1997). Undifferentiated U937 cells were harvested by centrifugation at 500 × *g* for 5 min, washed once with phosphate-buffered saline (Gibco-BRL, Burlington, Ont.), repelleted, and stored at –80°C until processing. Cells were thawed and resuspended in 0.1 M Tris-HCl, pH 7.4, 10 mM EDTA, 2 mg/mL leupeptin, 2 mg/mL soybean trypsin inhibitor, 2 mg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride (typically 1×10^{10} cells in 250 mL of buffer). The cell suspension was sonicated 4 times for 10 s (Cole Parmer Ultrasonic Homogenizer 4710, Cole Parmer Instrument Co.,

Chicago, Ill.; output control, 3.8; 70% duty cycle) and then centrifuged at 10 000 × *g* for 10 min at 4°C. The supernatant was centrifuged at 100 000 × *g* for 1 h at 4°C, and the resulting pellet was resuspended in 0.1 M Tris-HCl, pH 7.4, 10 mM EDTA. Aliquots of the resuspended pellet, referred to as the microsomal preparation (7–20 mg of protein/mL), were stored at –80°C. Protein concentrations were determined using the Bio-Rad (Mississauga, Ont.) Coomassie protein stain.

Immediately prior to use, microsomal preparations were thawed, subjected to a brief sonication, and then diluted to a protein concentration of 125 μg/mL in 0.1 M Tris-HCl, 10 mM EDTA, pH 7.4, containing 0.5 mM phenol, 1 mM reduced glutathione, and 1 μM haematin. Compounds were tested at 8 concentrations in duplicate using 3-fold serial dilutions in DMSO of the highest drug concentration. A 5-μL sample of test compound or DMSO vehicle was added to 20 μL of 0.1 M Tris-HCl, pH 7.4, 10 mM EDTA, in a 96-well polypropylene minitube plate (Beckman, Mississauga, Ont.) and mixed with 200 μL of the microsomal suspension. After a preincubation for 15 min at room temperature, 25 μL of a solution of 1 mM peroxide-free arachidonic acid (Cayman Chemical Co.) in 0.1 M Tris-HCl, pH 7.4, 10 mM EDTA, was added to give a final concentration of arachidonic acid of 0.1 μM. The samples were mixed and incubated at room temperature for 40 min. Control samples contained ethanol vehicle instead of arachidonic acid. Following the incubation period, the reaction was terminated by the addition of 25 μL of 1 M HCl. Samples were neutralized by the addition of 25 μL of 1 M NaOH prior to quantitation of PGE_2 by radioimmunoassay (NEN–DuPont, Boston, Mass., or Amersham, Oakville, Ont.). These procedures were automated using a Biomek 1000 (Beckman). Cyclooxygenase activity in the absence of test compounds is defined as the difference between PGE_2 levels in samples incubated in the presence of arachidonic acid versus the ethanol vehicle. The percentage of inhibition of PGE_2 synthesis is calculated from the difference between PGE_2 levels in samples incubated in the absence or presence of the test compounds.

Thromboxane B_2 production by calcium ionophore activated human platelets

Platelets were prepared from human venous blood obtained from healthy volunteers. The collected blood was immediately mixed with 1/10th volume of anticoagulant solution (65 mM citric acid, 85 mM sodium citrate, and 2% glucose) and centrifuged at 200 × *g* for 10 min. The supernatant was mixed with 50% volume of Hanks' balanced salt solution buffered with 15 mM Hepes, pH 7.4 (HHBSS), and 30% volume of the anticoagulant solution. This mixture was centrifuged at 750 × *g* for 10 min, and the pellet was resuspended in HHBSS. Platelet concentration was determined with a Coulter counter. Platelets were preincubated at a final concentration of 4×10^7 cells/mL (0.2–0.25 mL) in the absence or presence of the inhibitor (from a 125-fold concentrated solution in DMSO) for 15 min before stimulation with 2 μM calcium ionophore A23187. After a further 10-min incubation at 37°C, cold (4°C) methanol was added (50% by volume) to stop the reaction and thromboxane B_2 (TXB_2) levels were measured by enzyme immunoassay (Assay Designs Inc.). Inhibitors were tested at 8 concentrations using 3-fold serial dilutions of the highest drug concentration. Human platelets released 13–20 ng of $TXB_2/10^7$ cells following challenge with 2 μM A23187 and 4–10 ng $TXB_2/10^7$ cells when stimulated with 1 μM arachidonic acid instead of ionophore. Less than 5% of the total production of TXB_2 was observed in the absence of ionophore challenge.

Assays with transfected CHO cells expressing COX-1

Stably transfected CHO cells expressing human COX-1 were obtained from G.P. O'Neill (Merck Frosst). The production of PGE_2 by the CHO[COX-1] cells following stimulation by arachidonic acid was used as a cell-based assay for COX-1 as previously described (Kargman et al. 1996*b*) and as summarized below. CHO[COX-1] cells were washed once and resuspended in Hanks' balanced salts solution containing 15 mM Hepes, pH 7.4, at a cell concentration of 1.5×10^6 cells/mL. The cells were preincubated with test drug or DMSO vehicle for 15 min at 37°C before challenge with a final concentration of 0.5 μM arachidonic acid. After an incubation of 15 min at 37°C with arachidonic acid, the reaction was terminated by acidification. PGE_2 production was quantitated using an EIA (Correlate PGE_2 enzyme immunoassay kit, Assay Designs Inc., Ann Arbor, Mich.) or RIA (Amersham). The levels of PGE_2 in samples from CHO[COX-1] increased from <80 pg to 0.3–1.3 ng $PGE_2/10^6$ cells following stimulation with 0.5 μM exogenous arachidonic acid. Cyclooxygenase activity in the absence of test compounds is determined as the difference in PGE_2 levels of cells challenged with arachidonic acid versus the PGE_2 levels in cells mock challenged with ethanol vehicle. Each experiment included a set of 8 positive and negative control samples (± arachidonic acid challenge) for cells preincubated in the absence of inhibitor. Compounds were typically tested at 8 concentrations in duplicate using 3-fold serial dilutions in DMSO. Inhibition of PGE_2 synthesis by test compounds is calculated as a percentage of the activity in the presence of drug versus the activity in the positive control samples.

Results

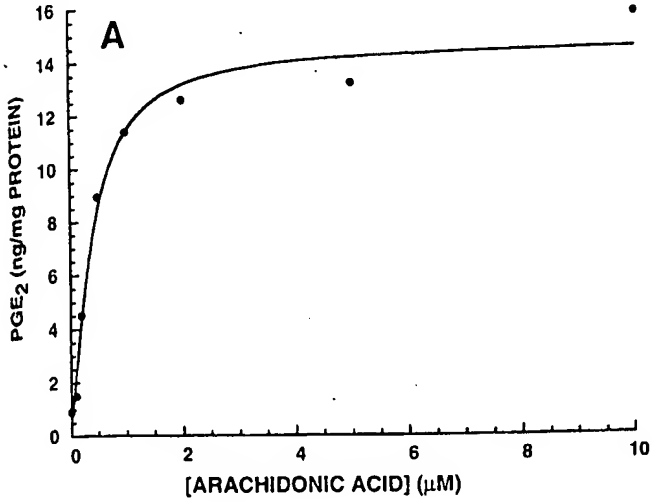
Measurement of COX-1 activity at low arachidonic acid concentration

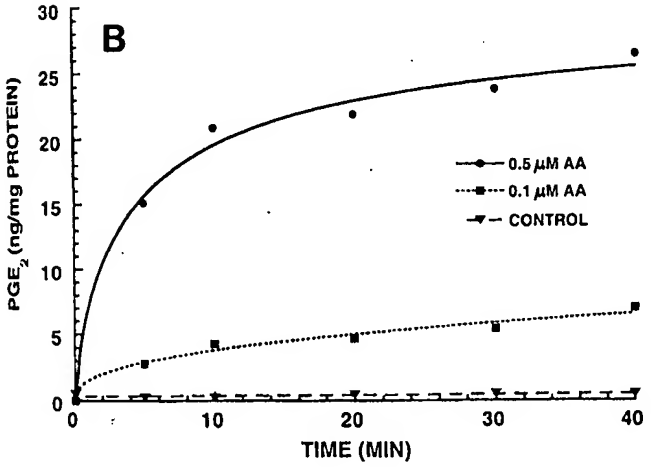
Microsomal membranes from U937 cells were used as a source of COX-1 and assayed for the arachidonic acid dependent production of PGE_2. The apparent K_m for arachidonic acid using this preparation was 0.6 μM (Fig. 1A). A significant stimulation of PGE_2 production was observed at the low concentrations of 0.1 and 0.5 μM arachidonic acid for which the time course of the reaction was measured over a 40 min period (Fig. 1B). An accumulation ranging from 3 to 12 ng PGE_2/mg protein for various preparations and an average 10-fold stimulation by arachidonic acid were measured using the 40-min reaction at 0.1 μM arachidonic acid. These conditions were selected for the evaluation of the inhibitory effects of compounds on the COX-1 reaction at a subsaturating concentration of arachidonic acid.

Effects of NSAIDs and COX-2 inhibitors on COX-1 activity assayed at low substrate concentration

The production of PGE_2 in the U937 microsome assay at low arachidonic acid can be inhibited dose dependently by NSAIDs and selective COX-2 inhibitors. Examples of inhibitor titrations showing that the nonselective flurbiprofen is about 300 and 10 000 times more potent at inhibiting microsomal COX-1 than the selective COX-2 inhibitors NS-398 and DFU, respectively, are given in Fig. 2. The inhibition by flurbiprofen was stereoselective, the (*S*)-flurbiprofen being 600-fold more potent than (*R*)-flurbiprofen (Table 1). The assay was very sensitive to inhibition and allowed the detection of inhibitory effects for each of the NSAIDs and selective COX-2 inhibitors tested (Table 1). IC_{50} values ranged from 0.6 nM for (*S*)-flurbiprofen to 500 μM for salicylic acid. L-745,296, a selective COX-1 inhibitor (Leblanc et al. 1995), was a very potent inhibitor of COX-1 under these conditions, with an IC_{50} value of 1.9 nM. The selective COX-2 inhibitors NS-398 (IC_{50} 0.3 μM), SC-58125 (IC_{50} 0.76 μM), L-745,337 (IC_{50} 2.8 μM), and DFU (IC_{50} 13 μM) were, respectively, 15-, 38-, 140-, and 640-fold less potent inhibitors of the microsomal COX-1 activity at low

Fig. 1. Production of PGE_2 by microsomes from U937 cells. (A) Dependence of PGE_2 production on arachidonic acid concentration, using a reaction time of 3 min. (B) Time dependence of the production of PGE_2, using initial arachidonic acid concentrations of 0.1 and 0.5 μM.





arachidonic acid concentration than indomethacin (IC_{50} 20 nM).

Inhibition of TXB_2 production by human platelets

The potency of NSAIDs and COX-2 inhibitors as inhibitors of Ca^{2+} ionophore induced TXB_2 production by human platelets was also determined (Table 1). Potent time-dependent inhibitors, such as flurbiprofen and indomethacin, showed IC_{50} values in the low nanomolar range, which is similar or slightly lower than those measured using the U937 microsomal assay. For selective COX-2 inhibitors such as DuP 697, NS-398, and DFU, which behave as reversible competitive inhibitors of COX-1, IC_{50} values were in general 2–10 times higher in the platelet than in the microsomal assay. A good correlation was observed between the IC_{50} values measured for the inhibition of PGE_2 production by microsomes at low arachidonic acid concentration and those determined for the inhibition of TXB_2 by calcium ionophore stimulated platelets (Fig. 3). Some deviation was observed with flufenamic acid and etodolac, which were found to be either less or more potent, respectively, in the

Fig. 2. Concentration dependence of the inhibitory effects of NSAIDs and COX-2 inhibitors on the production of PGE_2 by U937 microsomes. Inhibitors were preincubated with the enzyme for 15 min prior to incubation for 40 min with 0.1 μM arachidonic acid.

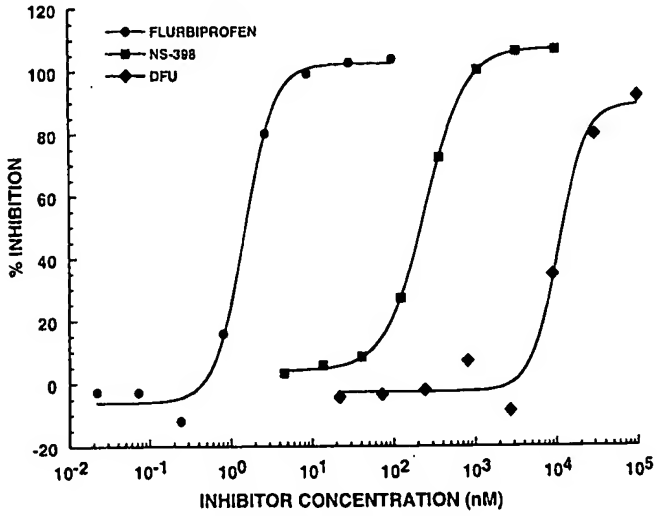
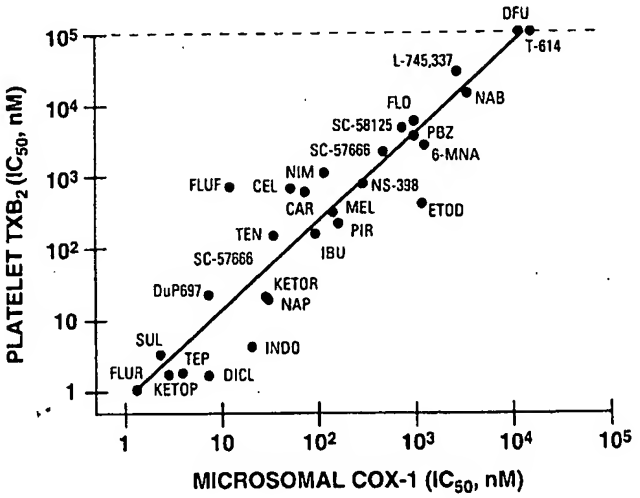


Fig. 3. Correlation between the IC_{50} values of the platelet and microsomal assays for COX-1. Compounds were tested for the inhibition of TXB_2 by calcium ionophore stimulated platelets and for inhibition of the production of PGE_2 by U937 microsomes at low substrate concentration. Data for the different NSAIDs and COX-2 inhibitor are from Table 1.

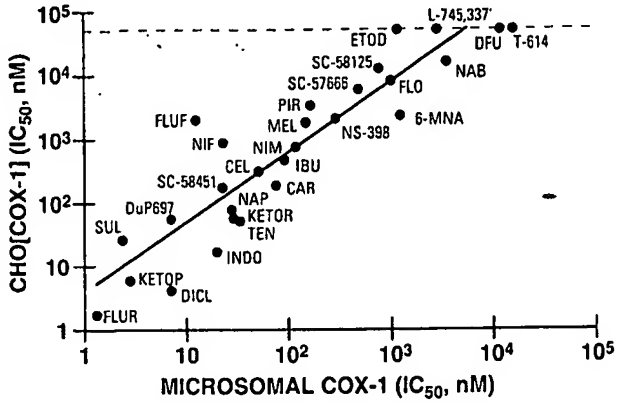


platelet assay than inhibitors of comparable potency in the COX-1 microsomal assay. The results suggest that the inhibition of COX-1 accounts for the effects of most of the inhibitors tested on TXB_2 synthesis by activated platelets.

Inhibition of PGE_2 production by CHO[COX-1] cells

The production of PGE_2 by CHO[COX-1] cells stimulated by arachidonic acid (0.5 μM) has been used previously as a cell-based assay for COX-1 (Kargman et al. 1996*b*). Comparison of the inhibition data obtained for the CHO[COX-1] cells with those of ionophore-challenged platelets (Table 1) indicates that certain inhibitors are more potent in the latter assay, with the largest differences observed for piroxicam and etodolac (16- and 120-fold, respectively). In general, the rank order of potency of the various inhibitors tested was similar to that of

Fig. 4. Correlation between the IC_{50} values for the inhibition of PGE_2 in the CHO[COX-1] cells and microsomal COX-1 assays. Data are from Table 1.



the other two assays for COX-1. The correlation between the IC_{50} values for the CHO[COX-1] and U937 microsomal assays is shown in Fig. 4.

Discussion

Several factors have been implicated in the mechanism of gastrointestinal intolerance to NSAIDs, including topical irritation, promotion of acid back-diffusion into the gastric mucosa, enterohepatic recirculation, uncoupling of oxidative phosphorylation, and inhibition of the synthesis of cytoprotective prostaglandins (Hudson et al. 1992; Whittle 1992). Prostaglandins of the gastrointestinal tract are mainly derived from COX-1 (Kargman et al. 1996*a*) and appear to protect the gastric mucosa by inhibiting acid secretion, raising bicarbonate output and mucous secretion, and by maintaining mucosal blood flow (Appleyard et al. 1996; Polisson 1996; Wallace 1994). There has been some discussion about the relative importance of the various mechanisms, but most investigators seem to regard the mechanism of NSAID toxicity as a multifactorial process, of which COX-1 inhibition is one important element (Bennett and Tavares 1995; Hayllar and Bjarnason 1995; Wallace 1994). The multiplicity of the factors involved in mucosal cytoprotection might also explain the absence of spontaneous gastrointestinal lesions in COX-1-deficient mice (Langenbach et al. 1995).

To develop a sensitive assay for the inhibition of COX-1, we have optimized an assay at low arachidonic acid concentration, using microsomes from U937 cells. This assay was found to be more reproducible and slightly more sensitive than that using the purified enzyme at the same arachidonic acid concentration, presumably as a result of membranes acting as a carrier and further diluting the arachidonic acid substrate. It was more sensitive to inhibition than assays using gastric mucosa homogenates (Boughton-Smith and Whittle 1983) or minced intestinal tissues (Futaki et al. 1992). The evaluation of inhibitory effects on COX-1 at low arachidonic acid may be especially relevant to those related to NSAID-induced gastropathy considering that the utilisation of arachidonic acid appears to be limiting in the gastric mucosa. For example, the intragastric administration of arachidonic acid has been shown to cause a 400-fold elevation of PGE_2 over basal levels (Doyle et al. 1989). In addition, the presence of arachidonic acid binding proteins, which reduce arachidonic acid utilisation by cyclooxygenase, has been demonstrated in microsomes from the gastric mucosa (Preclik et al. 1992).

It should be noted that the effect of lowering the arachidonic acid concentration on inhibitory potency will depend on the mechanism of inhibition of the particular compound tested. Three different mechanisms of inhibition have been elucidated for COX-1 inhibition (Griswold and Adams 1996). A first mechanism of inhibition involves the time-dependent formation of a tight enzyme–inhibitor complex and is typically observed for potent nonselective inhibitors such as indomethacin and flurbiprofen. The second mechanism is through a rapid reversible binding to the enzyme, competitive with arachidonic acid. Less potent inhibitors of COX-1, such as the selective COX-2 inhibitors DuP 697, NS-398, and DFU, fall into this category. Acetylsalicylic acid has a distinct irreversible mechanism involving a covalent acetylation of an active-site serine residue. In assays where inhibitors are preincubated with enzyme prior to the addition of arachidonic acid, lowering the substrate concentration will result in an increase in potency for competive inhibitors, whereas little effect should be observed for slowly reversible and irreversible inhibitors.

A good correlation was found between the inhibition of microsomal COX-1 and both the inhibition of TXB_2 synthesis by Ca^{2+} ionophore challenged platelets and the inhibition of PGE_2 production by CHO[COX-1] cells. The IC_{50} values in the platelet assay are about 10- to 300-fold lower than those reported for platelets stimulated with 5–10 μM arachidonic acid (Grossmann et al. 1995; Klein et al. 1994). The difference in potency might be explained by a higher effective concentration of arachidonic acid in the latter assay compared with ionophore-challenged platelets. In other experiments, we have observed a decrease in potency for several inhibitors by raising either the ionophore or arachidonic acid concentration (unpublished observations).

Long-term use of NSAIDs is associated with gastrointestinal side effects such as ulceration and bleeding (Champion et al. 1997; Langman et al. 1994). All NSAIDs tested were found to inhibit the synthesis of COX-1-derived PGE_2 in the U937 microsome assay at low arachidonic acid concentration. The assay allows the discrimination of inhibitor potencies over a wide range of IC_{50} values, ranging from 1 nM for flunixin and flurbiprofen to 200–500 μM for salicylate and acetaminophen. The latter two compounds are also very weak inhibitors of gastric PGE_2 production in vivo, acetaminophen being nonulcerogenic and salicylate having toxic effects on the gastric mucosa, which appears to be unrelated to the inhibition of prostanoid synthesis (Laporte et al. 1991; Whittle 1992). Acetylsalicylic acid was only slightly more potent than salicylate in the COX-1 microsomal assay. Acetylsalicylic acid is a known inhibitor of the production of gastric prostaglandins at high doses, and its potency was probably underestimated relative to other inhibitors under the present assay conditions, considering the irreversible mechanism of inhibition. Several of the currently marketed NSAIDs such as ketorolac, naproxen, piroxicam, nimesulide, and meloxicam had a potency similar to that of ibuprofen, with IC_{50} values 10 to 50 times higher than those of flurbiprofen, ketoprofen, or meclofenamic acid. Tepoxalin was among the most potent COX-1 inhibitors. The weak ulcerogenicity of this compound in rats has been related to dual inhibitory effects on the syntheses of prostaglandins and leukotrienes (Argentieri et al. 1994). Both the prodrug

Table 1. Effects of NSAIDs and COX-2 inhibitors on COX-1 activity from U937 cell microsomes, human platelets, and CHO[COX-1] cells.

	IC_{50} (nM)		
	U937 microsomes	Platelets	CHO[COX-1]
(*S*)-Flurbiprofen	0.6 ± 0.2 (3)	nd	0.5 ± 0.3 (2)
Flunixin	1.1 ± 0.1 (2)	nd	17 ± 5 (2)
Flurbiprofen	1.4 ± 0.3 (3)	1.1 ± 0.1 (2)	1.8 ± 0.4 (7)
L-745,296	1.9 ± 0.4 (2)	nd	31 (1)
Sulindac sulfide	2.3 ± 0.9 (2)	3.3 ± 1.1 (3)	28.0 ± 6.7 (10)
Ketoprofen	2.8 ± 0.3 (3)	1.7 ± 0.1 (2)	6.1 ± 0.1 (2)
Meclofenamic acid	3.8 ± 0.4 (3)	nd	1.8 (1)
Tepoxalin	3.8 ± 0.4 (2)	1.8 ± 0.3 (2)	<22 (2)
Diclofenac	7.0 ± 3.0 (3)	1.7 ± 0.1 (2)	4.3 ± 1.1 (5)
DuP 697	7.1 ± 3.4 (3)	21.9 ± 5.9 (3)	59 ± 14 (8)
Flufenamic acid	12.1 ± 3.3 (3)	700 ± 330 (2)	2 100 ± 960 (10)
Indomethacin	19.8 ± 0.2 (23)	4.8 ± 1.1 (4)	17.6 ± 3.1 (7)
SC-58451	23.0 ± 1.6 (3)	nd	180 ± 110 (2)
Niflumic acid	23.3 ± 0.8 (3)	nd	950 ± 250 (4)
Zomepirac	24.2 ± 1.1 (3)	nd	nd
Ketorolac	28.0 ± 6.5 (3)	21.3 ± 9.9 (2)	82.9 ± 0.8 (3)
Mefenamic acid	28.8 ± 8.3 (2)	nd	nd
Naproxen	29.4 ± 7.5 (3)	19.7 ± 5.8 (3)	62 ± 29 (3)
Tolmetin	30.1 ± 3.3 (2)	19.5 ± 1.6 (2)	157 (1)
Tenidap	34 ± 13 (4)	144 ± 56 (3)	55 ± 27 (2)
Fenoprofen	47.1 ± 0.7 (2)	nd	nd
Celecoxib	52.3 ± 8.7 (3)	660 ± 20 (2)	320 ± 120 (2)
Carprofen	75.9 ± 2.0 (2)	610 ± 140 (4)	200 ± 120 (2)
Ibuprofen	94 ± 13 (3)	154 ± 3 (2)	470 ± 60 (8)
Nimesulide	117 ± 37 (3)	1 110 ± 440 (4)	780 ± 220 (3)
Meloxicam	143 ± 55 (2)	310 ± 110 (3)	1 810 ± 430 (4)
Piroxicam	163 ± 17 (3)	210 ± 130 (3)	3 460 ± 990 (4)
Tenoxicam	229 ± 71 (2)	nd	nd
RS-57067	287 ± 8 (2)	1 900 ± 400 (3)	3 580 ± 860 (2)
NS-398	300 ± 120 (4)	780 ± 310 (5)	1 930 ± 610 (12)
Fenclofenamic acid	316 ± 35 (3)	nd	nd
(*R*)-Flurbiprofen	390 ± 55 (3)	nd	620 (1)
SC-57666	480 ± 130 (3)	2 100 ± 200 (3)	6 000 ± 1 900 (2)
SC-58125	762 ± 68 (3)	4 800 ± 1 400 (3)	12 300 ± 8 700 (3)
Flosulide	1 005 ± 70 (3)	6 000 ± 1 700 (4)	8 100 ± 3 700 (3)
Phenylbutazone	1 012 ± 77 (2)	3 700 ± 1 700 (4)	8 000 ± 1 800 (2)
BW 755C	1 010 ± 210 (3)	nd	nd
Etodolac	1 200 ± 260 (4)	400 ± 110 (3)	~50 000 (5)
6-MNA	1 300 ± 600 (6)	2 800 ± 1 800 (6)	2 290 ± 530 (4)
Isoxicam	1 320 ± 190 (2)	nd	4 200 (1)
L-745,337	2 780 ± 280 (3)	29 000 ± 19 000 (5)	~50 000 (5)
Nabumetone	3 530 ± 730 (3)	14 100 ± 5 500 (5)	15 700 ± 2 700 (2)
Azapropazone	3 800 ± 1 200 (4)	nd	nd
Benoxaprofen	3 840 ± 780 (2)	nd	nd
DFU	12 600 ± 2 400 (11)	> 100 000 (8)	> 50 000 (6)
T-614	16 700 ± 4 800 (4)	> 40 000 (3)	> 50 000 (2)
Sulindac sulfoxide	15 600 ± 5 100 (5)	nd	nd
Acetylsalicylic acid	21 500 ± 6 400 (3)	nd	nd
Sulindac sulfone	46 900 ± 7 800 (5)	nd	nd
Acetaminophen	188 000 ± 59 000 (3)	nd	~ 50 000 (2)
Salicylic acid	490 000 ± 120 000 (3)	nd	nd

Note: Compounds were tested using a 15-min preincubation in the indicated assay. IC_{50} values are given ± range ($n = 2$) or SE ($n > 2$); nd, not determined.

nabumetone and its active metabolite 6-MNA (Blower 1992), a rather nonselective cyclooxygenase inhibitor (Grossmann et al. 1995), were found to inhibit the microsomal COX-1. Sulindac sulfone and sulindac sulfoxide also caused inhibition at high concentrations but were considerably less potent than the active sulindac sulfide form.

Inhibition of COX-1 at low substrate concentration was detected with all selective COX-2 inhibitors tested, including

DuP 697, NS-398, SC-58125, and L-745,337, for which anti-inflammatory effects have been demonstrated at doses that are non-ulcerogenic and that do not inhibit gastric PGE_2 production (Chan et al. 1995; Futaki et al. 1994; Gans et al. 1990; Masferrer et al. 1994; Seibert et al. 1994). Among the compounds for which in vitro selectivity for COX-2 has been reported, the rank order of potency against COX-1 was DuP 697 > SC-58451 > celecoxib > nimesulide ~ meloxicam ~ piroxicam ~ NS-398 ~ RS-57067 > SC-57666 > SC-58125 > flosulide > etodolac > L-745,337 > DFU ~ T-614. For this class of compounds, it is expected that the selectivity ratio of COX-2/COX-1 inhibition will be of primary importance to determine the effective dose as anti-inflammatory and analgesic agent versus the dose responsible for gastrointestinal side effects. For example, T-614, which was found to be about 300-fold less potent than celecoxib in the COX-1 microsomal assay, is also about 100-fold less potent as a COX-2 inhibitor in the CHO[COX-2] assay (unpublished observations). Gastrointestinal toxicity has been observed at high doses with meloxicam, piroxicam, flosulide, and etodolac (Engelhardt et al. 1995; Melarange et al. 1995; Wiesenberg-Bottcher et al. 1989) and may be related to COX-1 inhibition. No detectable loss of the integrity of the gastrointestinal tract was observed with DFU administered at a dose 200-fold higher than the efficacious anti-inflammatory dose (Riendeau et al. 1997). Obviously, the ability of these inhibitors to block COX-1 activity in vivo will depend on a number of different factors, including oral bioavailability, tissue distribution, binding to protein, and pharmacokinetics. Therefore, the ulcerogenic potential of a compound cannot be predicted simply on the basis of its potency as an inhibitor of COX-1 in vitro. Nevertheless, the microsomal assay at low substrate concentration is particularly useful to compare relative potencies of weak inhibitors of COX-1.

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